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Correlation between *Aspergillus flavus* fungal biomass and aflatoxin contamination in harvested maize: insights from Kenya and Tanzania

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ABSTRACT

Controlling the occurrence of aflatoxins in foods must be accompanied by managing the fungi responsible for their production. The abundance and diversity of aflatoxin-producing *Aspergillus flavus* are responsible for the accumulation of these toxins in crops, posing a persistent threat to public health and the economy in tropical developing countries. A study was conducted to investigate the occurrence and level of *A. flavus* and relate them to aflatoxin levels in maize in Kenya and Tanzania. A total of 786 maize samples were collected during harvesting in selected areas of the two countries for analysis. The fungal abundance in the samples was measured as the amount of fungal DNA relative to maize DNA. This was accomplished by quantifying the fungal DNA using qPCR, targeting the internal transcribed spacer (ITS) gene, while the maize DNA was quantified through the alpha-tubulin gene, the two genes known to be conserved. Aflatoxins were quantified using ultra-high performance liquid chromatography, coupled with ultra-high sensitivity, ultra-fast triple quadrupole tandem-mass spectrophotometer. *A. flavus* was detected in 88.5% of the 786 tested samples, and the average fungal load for these samples (expressed as the log host/pathogen ratio) was 5.53. Aflatoxin occurrence was positive in 31.9% of the samples, with an average level of 2.3 ± 0.643 ppb. The study established a positive relationship between the occurrence and level of aflatoxin B_1 and the presence and biomass of *A. flavus*, which was statistically proven. These findings emphasize the need to place substantial attention on preharvest control of *A. flavus* in cereal fields as an effort to control the accumulation of aflatoxin B1 in foods.

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1. Introduction

Aspergillus flavus is a fungus that grows as a soil-borne saprophyte and commonly proliferates in plants and other organic products, including food and feed (1).

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Under favorable conditions, the fungus converts its primary metabolites into toxic substances called mycotoxins (2,3). Major mycotoxins which can affect human and animal health include aflatoxins, fumonisins, ochratoxins, zearalenone, deoxynivalenol, and T-2 toxin (4).

Among these, the most toxic are produced by the Aspergillus genus, particularly *A. flavus* and *A. parasiticus* and they include aflatoxins, primarily aflatoxin B1, B2, G1, and G2 (5). The number of mycotoxins that accumulate in food and feed has been indicated to be associated with the growth and development of mycotoxin-producing fungi (6). The most affected foods are grains, especially maize, and peanuts (7). The toxins accumulate also in other crops and products including spices (8), oily nuts like pistachio (9) and sunflower, and legumes (10).

Mycotoxins exhibit varying degrees of health impacts on both humans and animals, with liver cancer being among the severe consequences. Aflatoxin B1, recognized as the most potent naturally occurring carcinogen, has been assigned to class 1A by the International Agency for Research on Cancer (11). In developed regions like Europe, stringent control regulations have been put in place (12), resulting in a significant reduction of mycotoxin exposure in both humans and animals. However, these regulations also come with considerable financial costs. In the United States, mycotoxin-related losses in the food and feed sectors are estimated to reach billions of US dollars annually, primarily due to the disposal of contaminated products (13).

Nevertheless, in regions with prevalent informal business chains, the implementation of regulatory measures to control mycotoxin exposure to consumers is less common (14). Studies indicate that countries with a high occurrence of mycotoxins in foods tend to experience above-average rates of liver cancer and stunted growth compared to the global average (15-17).

Efficient control of aflatoxin occurrence in maize relies heavily on precise targeting, which should consider the fungal presence in the crops. Assessing the presence of *A. flavus* at harvest not only reveals the current situation of aflatoxin contamination but also indicates the risk of elevated toxin levels during storage (18). *A. flavus* conidia present in the soil pose a threat in the maize field, as they can infect the maize through its silk, multiply, and then invade the maize cob to colonize the kernels (19). To counteract this, preventive measures such as employing curcumin-mediated photosensitization to neutralize *A. flavus* (20) and investigating the potential of *Candida albicans* (21) have shown promise in achieving the desired objective. Moreover, the production of aflatoxin by contaminating Aspergillus species can be addressed through the biocontrol effect of *Kluyveromyces lactis* (22) or the inhibitory effect of lactic acid bacteria (23). These approaches offer potential solutions to address the issue of aflatoxin contamination in maize.

To detect and identify fungal infections in maize kernels, various laboratory methods are commonly used, including culturing for phenotypic characterization, near-infrared radiation, and PCR-based molecular identification (24,25). Among these methods, PCR-based fungal identification, particularly through amplification of the internal transcribed spacer 1 (ITS1) region, has demonstrated superior specificity across different fungal species (26). This region is known to contain highly conserved ribosomal RNA genes that exhibit high specificity for different fungal species in which it occurs.

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A. flavus exhibits two types of strains, namely producers and non-producers, concerning aflatoxin B1 production (27). While it is feasible to isolate these strain types using laboratory culture techniques, their simultaneous presence in crop lots poses challenges in determining the exclusive absence of either type in a particular sample.

The intricate nature of the aflatoxin biosynthesis pathway and its associated genes creates challenges in using PCR for conclusive molecular identification of aflatoxin producers and non-producers (28-30). A. flavus strains capable of producing aflatoxins are frequently encountered in maize, particularly in Africa, and this fungus is known to produce various other toxins as well (31,32).

Mideros and others (33) established a correlation between A. flavus fungal biomass and aflatoxin B1 levels in maize kernels under laboratory conditions. This discovery holds importance as it suggests that quantitative analysis of the fungi could serve as a potential alternative to directly measuring the toxins. However, when it comes to field conditions, the accuracy of linking fungal biomass and aflatoxin levels requires consideration of other natural flora that might influence the observed patterns. In our study, we aimed to investigate whether aflatoxin levels in maize, contaminated under natural field conditions, are correlated with fungal biomass determined through quantitative real-time PCR.

2. Materials and Methods

2.1. Study area and design

The study was conducted using a cross-sectional survey design. We gathered maize samples from small-scale farmers with maize stands in various regions of Kenya and Tanzania. In Tanzania, a total of 300 samples were collected from the administrative regions of Kagera, Kigoma, Mbeya, Morogoro, Mwanza, and Tanga. In Kenya, we collected 478 maize samples from the Central region, Coastal region, Eastern region, Nairobi, Nyanza, Western region, and Rift Valley. During the harvest, dried maize cobs were obtained as the samples, and later, they were de-hulled. These maize samples were stored under refrigeration at agricultural research centers before being transported to a central laboratory for analysis.

2.2. Sample preparation

The samples underwent sieving and milling using a Romer Series II® miller. The milled maize samples were then stored at 4°C in sealed plastic bags, and prepared for DNA and mycotoxin extraction and quantification.

2.3. Maize and fungal DNA

Quantification of DNA was performed using qPCR, targeting the internal transcribed spacer (ITS) gene for A. flavus and the alpha-tubulin gene for maize. Both of these genes are known to be conserved in their respective species. To create standard curves, pure maize DNA extracted from maize leaves was used, while pure A. flavus DNA extracted from fungal mycelia was utilized. Genomic DNA extraction was carried out using a simplified CTAB-based method (34) and the method by Porebski (35) with slight modifications.

2.4.DNA amplification by PCR

The *A. flavus* and maize-specific primers were referenced from a previously reported method by Mideros (33). The primers used and the amplified

products are summarized in Table 1. Both amplifications employed SYBR-green as an indicating dye. The optimal PCR conditions for each primer were determined using gradient PCR with AccuPower® Taq PCR premix on Techne TC-Plus® (UK) thermocyclers, and these conditions are summarized in Table 2.

For the creation of standard curves, *A. flavus* DNA was diluted in reagent sterile water at concentrations of 40, 4, 0.4, 0.04, 0.001, and 0.0004 ngµl-1. Host DNA was also diluted at concentrations of 50, 5, 1, 0.5, 0.05, 0.005, and 0.0005 ngµl-1. The qPCR was carried out using Applied Biosystems® 7500 Real-Time PCR Systems.

2.5. Standard curves, Primer specificity, DNA purity, and reproducibility assays

To assess the specificities of the Zmt3 and Af2 primers to the extracted DNAs, separate PCR runs were performed using each primer with maize and *A. flavus* DNAs individually, as well as with the DNAs mixed. The same DNAs used for generating the standard curves were utilized in this analysis. This step was crucial in ensuring that there was no cross-contamination between the DNAs intended for the Zmt3 and Af2 standard curves. To test the reproducibility of the method, qPCR was conducted on four different DNA extracts with predetermined concentrations in two separate runs. This allowed us to evaluate the consistency and reliability of the results obtained from the qPCR analysis.

2.6. Mycotoxin quantification by LC/MS/MS

Aflatoxin B1 was analyzed using ultra-high performance liquid chromatography, coupled to an ultra-high sensitivity, ultra-fast triple quadrupole tandem-mass spectrophotometer.

2.7. LC/MS/MS Method validation

Aflatoxin B1 standard was obtained as a concentrated solution with approximately 21 ngµl^{-1} of AFB1, from Sigma–Aldrich (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Working solutions were then prepared with concentrations of 21.0, 10.5, 5.25, 2.12, 1.06, 0.53, and 0.26 ngµl^{-1} and were stored at -20°C and allowed to reach room temperature before use. The sample extraction procedure was carried out by the method outlined in the literature (36). To assess the validity of the extraction method and chromatographic performance, a maize sample was spiked with three different concentrations (1, 3, and 5 ppb) of the standard AFB1 solution. The spiked samples were then subjected to extraction and analysis for the toxin in triplicate, and the average concentrations and percentage recovery were recorded. Additionally, a blank sample was included as a control. To determine the limit of detection (LOD) and limit of quantitation (LOQ) of the LC/MS/MS method for AFB1, the following equations were used:

LOD = Mean + 3 SD of the lowest concentration or blank

LOQ = Mean + 10 SD of the lowest concentration or blank

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Table 1. Primers used, target gene and product size

Genome	Targeted gene	Primers		Product size
		Name	Sequence	
Fungus (*A. flavus*)	Alpha-tubulin gene	Af2	Forward: 5′- ATCATTACCGAGTGTAGGGTTCCT-3′ Reverse: 5′- CCGAAGCAACTAAGGTACAGTAAA-3′	62 bp
Maize (*Zea mays*)	ITS* gene	Zmt3	Forward: 5′- TCCTGCTCGACAATGAGGC-3′ Reverse: 5′ - TTGGGCGCTCAATGTCAA-3′	73 bp

*Internal transcribed spacer

Table 2. PCR conditions for fungal Af2 and maize Zmt3 genes amplification

Condition	PCR					
	Alpha-tubulin gene (Af2)			ITS gene (Zmt3)		
	Temp (°C)	Time	Cycles	Temp (°C)	Time	Cycles
Annealing	57	-	-	60	-	-
Holding stage 1	50	2 min	-	50	2 min	-
Holding stage 2	95	10 min	-	95	10 min	-
Amplification	95 57	30 s 1 min	40	95 60	20 s 1 min	40
Dissociation stage 1	95	15 s	-	95	15 s	-
Dissociation stage 2	60	1 min	-	60	1 min	-
Dissociation stage 3	95	30 s	-	95	30 s	-

Each reaction was prepared in 20 µl with 10 µl of SYBR® Green Master mix, 1 µl forward primer (5 pg), 1 µl reverse primer (5 pg), and 3 µl of template DNA

3. Results

3.1. Maize and fungal DNA quantity

Reproducibility and standard curve for maize DNA

Fig. 1 displays the amplification plot and standard curve generated by qPCR using maize standard DNA extracted from maize leaves at concentrations of 5 ngµl^{-1}, 0.5 ngµl^{-1}, 0.05 ngµl^{-1}, 0.005 ngµl^{-1}, and 0.0005 ngµl^{-1}. The standard curve (Fig. 1) illustrates the relationship between DNA concentration and changes in fluorescence. The linear relationship between the Ct-values (threshold of 0.028753) and the initial DNA standard concentrations was shown with an R^2 of 0.98 (Fig. 2).

Reproducibility and standard curve for fungal DNA

Fig. 3 and 4 depict the standard curve and amplification plot generated by qPCR of the fungal DNA at concentrations of 20 ngµl^{-1}, 2 ngµl^{-1}, 0.2 ngµl^{-1}, 0.02 ngµl-1, 0.002 ngµl^{-1}, and 0.0002 ngµl^{-1}. The standard curve (Fig. 3) demonstrates a linear relationship between the standard concentration and Ct-value (threshold of 0.030403), measured by changes in fluorescence, with an R2 of 0.99.

3.2. LC/MS/MS method quality

Upon calculation, the limit of detection (LOD) was determined to be 0.16 ngµl-1, and the limit of quantitation (LOQ) was found to be 0.29 ngµl-1. The average recovery rate, as presented in Table 3, was 101.18%.

3.3. Occurrence and fungal load of *A. flavus* in the maize samples

A. flavus was found in 88.5% of the 786 samples tested. The relative fungal load was calculated by dividing the quantity of maize DNA by the quantity of fungal DNA, resulting in the host/parasite ratio for each maize sample. A lower ratio indicates a higher fungal load in the sample, while a higher ratio indicates a lower fungal load. The average fungal load for the samples, represented as the log host/pathogen ratio, was 5.53.

Fungal presence versus aflatoxins levels

Aflatoxin B1 was detected in 31.9% of the samples tested, with an average concentration of 2.3 ± 0.643 ngµl-1 (ranging from 0.00 to 1080 ngµl^{-1}). The aflatoxin B1 levels were categorized as high (≥ 5 ngµl^{-1}) or low (< 5 ngµl^{-1}) and then compared in terms of the likelihood of detecting *A. flavus*. The criteria for categorization were based on the acceptable levels for the study countries, Kenya and Tanzania. Among all the samples positive for AFB1, 39.8% had levels above the limit. When comparing fungal positive and fungal negative samples, 38.1% of AFB1 positive samples in the fungal positive group had levels above the limit, and 37.5% of AFB1 positive samples in the fungal negative group had levels above the limit. The ratios of samples with levels above the limits were not significantly different among the three groups.

3.4. Fungal biomass level versus aflatoxins presence

The fungal biomass was classified into three groups based on the maize/fungal DNA ratio: low occurrence (ratio < 4.0), medium occurrence (ratio between 4.0 and 5.9), and high occurrence (ratio above 5.9). In the AFB1 negative samples, the distribution of fungal levels was as follows: 45.5% were classified as low occurrence, 37.8% as medium occurrence, 2.4% as high occurrence, and the rest had undetectable levels. For AFB1 positive

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samples, the distribution was 5.2% undetectable, 19.4% low occurrence, 27.8% medium occurrence, and 47.7% high occurrence of *A. flavus*.

Comparing the occurrence of the three categories between AFB1 positive and AFB1 negative groups, a significant difference ($p<0.05$) was observed. Fig. 5 illustrates the distribution of fungal mass occurrence levels expressed by the log maize/fungal DNA ratio in the AFB1 positive and negative samples. The average log maize/fungal DNA ratio for samples positive for AFB1 was 4.4 ± 1.8, whereas, for negative samples, it was 6.1 ± 1.3. When tested as independent groups using ANOVA, the two levels showed a significant difference ($p<0.05$).

3.5. Fungal biomass versus aflatoxins level

The analysis revealed a negative correlation ($R2 = 0.596$) between the maize/fungal DNA ratio and the levels of AFB1 (Fig. 6). This suggests a positive relationship between fungal biomass and aflatoxin levels.

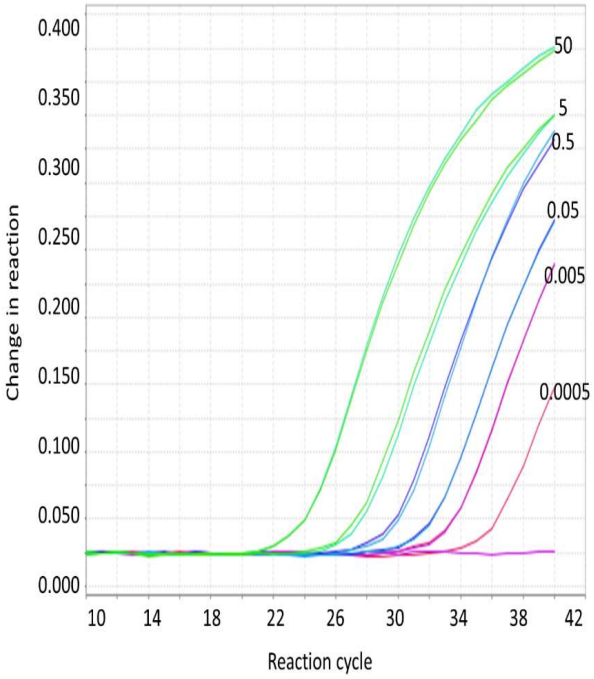

Figure 1. Mazie Standard DNA amplification curve

Table 3. Amount and percentage recovery of spiked aflatoxin B_1 standard in maze sample

Spiked		Replicate 1	Replicate 2	Replicate 3	Average
Blank	Recovered	0	0	0	
	%	0	0	0	0
1 ngµl^{-1}	Recovered	4.87	5.75	4.41	
	%	97.44	114.94	88.16	100.18
3 ngµl^{-1}	Recovered	5.56	4.80	5.77	
	%	111.24	95.96	115.38	107.5267
5 ngµl^{-1}	Recovered	5.08	4.66	4.64	
	%	101.56	93.19	92.76	95.83667
Average					**101.18**

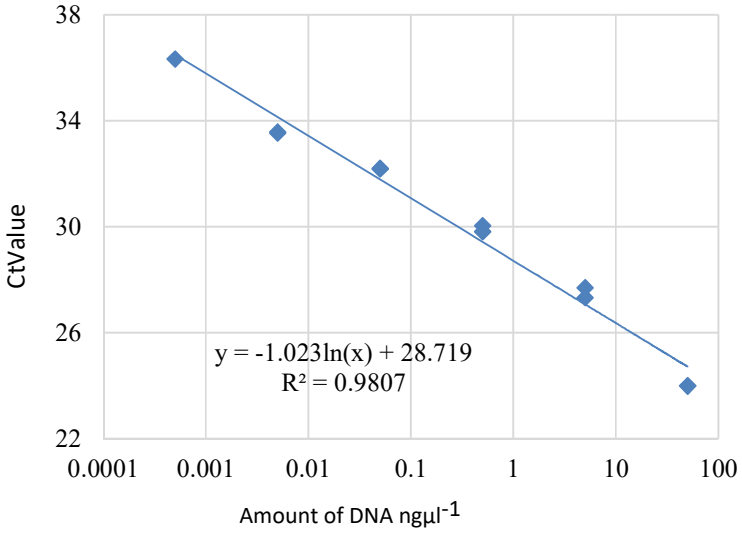

Figure 2. Maize DNA standard curve

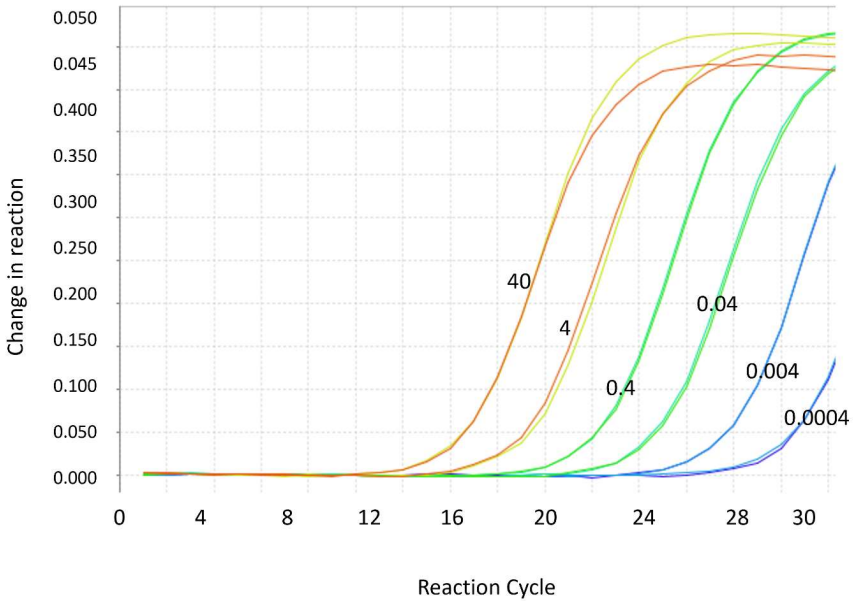

Figure 3. A. flavus DNA standard amplification plot

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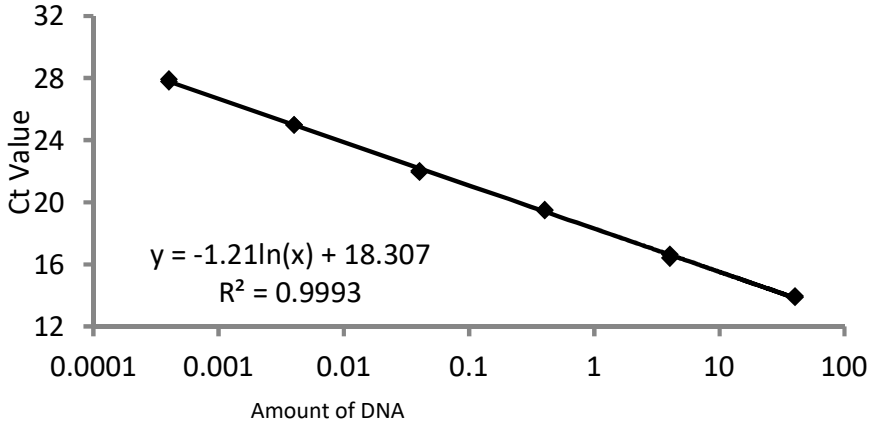

Figure 4. A. flavus DNA standard curve

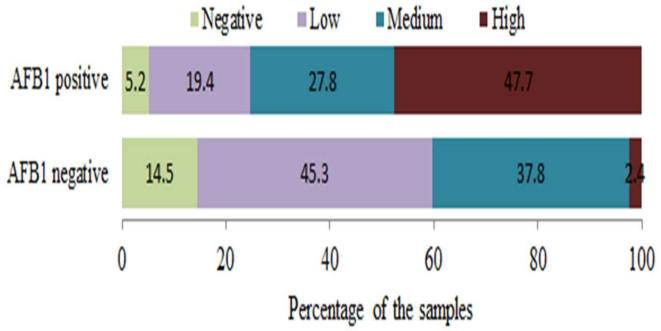

Figure 5. Distribution of fungal biomass frequencies in relation to aflatoxin B1 occurrence status

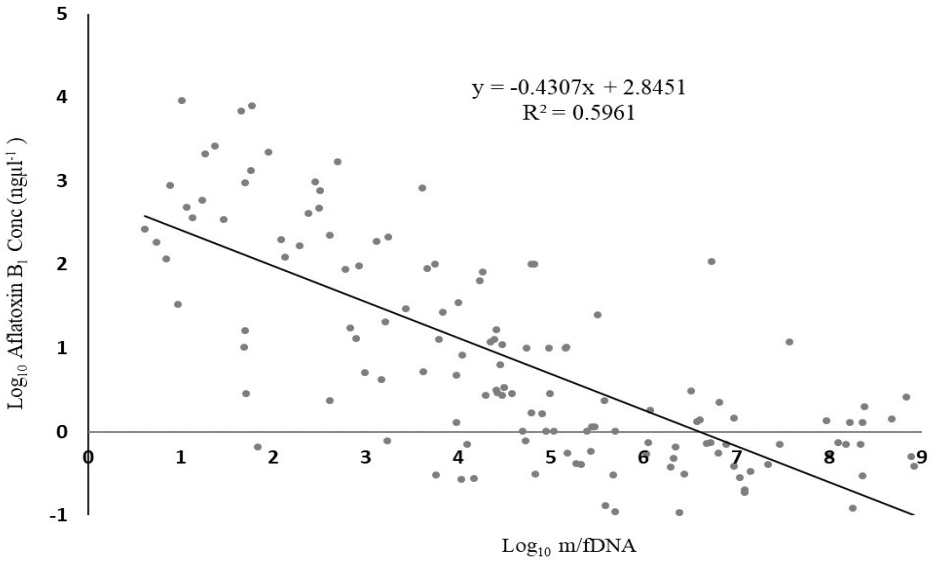

Figure 6. Correlation between fungal biomass and aflatoxins

4. Discussion

The present study reveals a high prevalence of fungi in maize samples, along with a moderate level of aflatoxin contamination, which falls below the regional accepted limits for contamination.

The discrepancy between the high fungal prevalence and the absence of high toxin prevalence could be attributed to the timing of sample collection and the specificity of the fungal detection method employed. The samples were targeted at or around harvesting, which might explain the elevated presence of *Aspergillus flavus*, the fungus responsible for aflatoxin production, but not significantly high levels of aflatoxins themselves.

Previous research has indicated that most fungal infestations occur in the field, while considerable mycotoxin production takes place during storage (37). Experimental conditions have demonstrated a positive correlation between inoculum size, fungal growth, and biomass with the accumulation of aflatoxins (33,38).

A. flavus strains that do not produce aflatoxins are commonly isolated from soils and crops (39). Previous reports have suggested that strains with low toxigenic potential are the ones most likely to transfer to storage agroecosystems (40). However, this study has revealed a positive correlation between naturally field-transmitted *A. flavus* and the presence of aflatoxin B1 in maize. These findings align with other studies conducted in Kenya and Tanzania, which have linked high occurrences of the fungi with aflatoxin production (41-43). This draws attention to the significance of field fungal infection and toxin accumulation in maize crops. In the natural environment, aflatoxin-producing strains make up over half of the total *A. flavus* population (30,44). Although our study does not specifically identify toxin producers, the observed correlation between the fungal presence and biomass with aflatoxin B1 accumulation suggests that toxigenic strains are present in a significant proportion within the infected maize.

Certain aflatoxin B1-positive samples tested negative for *A. flavus*, suggesting the potential involvement of other species in producing the toxin. *A. parasiticus* and *A. nonius* emerge as plausible candidates responsible for the detected aflatoxin B1 (44- 46). Nevertheless, in samples where *A. flavus* was present, the occurrence of aflatoxin B1 was significantly higher compared to samples where the fungus was absent, providing a strong indication that *A. flavus* is primarily responsible for toxin production. These findings align with previous studies that have indicated *A. flavus* as the main producer of aflatoxin B1 in grains under natural environmental conditions (1,47).

The present study has established a positive correlation between fungal biomass and aflatoxin B1 levels in maize. Similar findings have been demonstrated in multiple earlier studies (33,48). While most studies have explored the relationship between fungal infection, biomass accumulation, and mycotoxin levels, our study goes further by examining naturally infected maize in the field and testing it at harvest. This highlights the importance of efforts to combat aflatoxin B1 by reducing field fungal infestation to ensure cleaner products at harvest.

Despite the relatively lower number of samples with high levels of aflatoxin B1 and *A. flavus*, the significance of the observation lies in the fact that these samples were collected from family-held farms. Traditionally, in Tanzania and Kenya, most farmers store their harvest as food until the next harvest. Awareness of farmers

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and maize traders on proper handling of the grains during storage to reduce the chances of aflatoxin accumulation has been indicated to be insufficient (49). Therefore, even though the proportion is smaller, there is still a considerable number of families consuming foods that are highly contaminated with aflatoxin B1. Furthermore, the majority of the tested samples indicate the presence of *A. flavus*. This suggests that the levels and extent of toxins observed in this study will likely increase at some point during storage, thereby increasing the risk of human and animal exposure to aflatoxins and the associated health consequences.

5.Conclusion

In this study, a higher prevalence of *A. flavus* was observed in harvested maize from Kenya and Tanzania. The presence and quantity of *A. flavus* in the harvested maize correlated with the presence and quantity of aflatoxin B1. Notably, the average aflatoxin B1 level in both countries was found to be below 10 ppb.

Conflict of Interests

The authors declare no conflict of interest in this manuscript.

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